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Construction and *in Vivo* Efficacy of a Replication-Deficient Recombinant Adenovirus Encoding Murine Growth Hormone

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ABSTRACT

We have constructed a recombinant, replication-deficient, first-generation adenovirus-encoding mouse GH (mGH), AdCMVmGH. This virus directed mGH production from an epithelial cell line *in vitro* in a dose-dependent manner. When injected into the quadriceps muscle or submandibular ducts of mGH-deficient Snell dwarf mice, AdCMVmGH resulted in the production of significantly elevated serum mGH levels. Furthermore, after im injection, dwarf mice increased in weight by 8% over 4 days and close to 100% by 30 days. When AdCMVmGH was administered to 3- to 4-week-old rats by iv injection to assess general metabolic responses, serum mGH, insulin-like growth factor 1, triglycerides and cholesterol levels were significantly elevated. AdCMVmGH should be a valuable experimental tool for the controlled, directed expression of mGH in preclinical mouse model studies. (*Endocrinology* **140:** 260–265, 1999)

THE MANAGEMENT of numerous systemic protein deficiencies (*e.g.* diabetes, hemophilia A, GH deficiency) has been greatly facilitated by applications of molecular biological methods for the production of large amounts of pure, recombinant hormones. Though the repetitive injection of recombinant hormones circumvents concerns over coadministration of pathologic contaminants of biochemically-purified proteins from natural sources, this treatment approach is still viewed as inconvenient and is certainly not ideal (1, 2). Among the many approaches being attempted to improve the management of patients with systemic protein deficiencies is direct *in vivo* gene transfer to both somatic and germ line cells (3–5). The stable transfer of a gene encoding the deficient protein could lead, in principle, to the correction of the clinical defect (6).

Thus far, no *in vivo* gene transfer approach has replaced the conventional repetitive injection mode of clinical management. However, considerable progress has been made, and the results of several recent animal model studies are quite encouraging (4, 5). The most frequently used recombinant viruses in such studies are replication-deficient adenoviruses. In their studies of models of erythropoietin-responsive anemias using recombinant adenoviruses, Leiden and colleagues have noted the importance of using a transgene that encodes a protein identical to the test species' native protein (7, 8). For example, in immunocompetent mice injected with an adenovirus encoding murine erythropoietin, transgene expression dramatically improved over that seen when mice were injected with an adenovirus encoding human erythropoietin (7).

We have made a substantial effort to explore the therapeutic potential of reengineering exocrine salivary glands to secrete proteins in an endocrine fashion (9). Salivary glands are a convenient, if not often considered, site for *in vivo* gene transfer, because their excretory ducts can be readily cannulated in the mouth. One target disease that we have chosen to study is GH deficiency (10). The present series of experiments represents an extension of these efforts. The most widely used animal model for GH deficiency is the Snell dwarf mouse (11–13). These mice have a mutation on chromosome 16 and are unable to express Pit-1, a transcription factor necessary for the production of several anterior pituitary hormones and for the GH-releasing factor receptor (11, 12). Herein, we describe the construction of a replication-deficient, recombinant adenovirus encoding murine GH and initial demonstration of its efficacy and potential utility, through *in vivo* experiments.

Materials and Methods

Adenovirus construction

From the plasmid pKL-mGH (which was a generous gift of Dr. P.L. Chang, McMaster University, Hamilton, Ontario, Canada), a 750-bp

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fragment containing the mouse GH (mGH) complementary DNA (cDNA) was obtained by digestion with *Sal*I and *Hin*dIII (13). This fragment was then cloned into the *Sal*I and *Hin*dIII sites of the plasmid pACCMV-pLpA (a generous gift of Dr. C. Newgard, University of Texas-Southwestern Medical Center, Dallas, TX). The resultant vector, pACCMVmGH (Fig. 1), contains the cytomegalovirus (CMV) promoter-enhancer, and the SV40 polyadenylation sequence, and was subjected to DNA sequencing to confirm nucleotide sequences in the two joining regions. Thereafter, pACCMVmGH was cotransfected with pJM17 (a generous gift of Dr. F. Graham, McMaster University) into 293 (human embryonic kidney) cells, as previously described (10, 14). The recombinant virus so generated was plaque-purified and named AdCMVmGH.

In vitro infection of cells

AdCMVmGH was used to infect SMIE cells, an epithelial cell line derived from adult rat submandibular gland (15). SMIE cells were grown in DMEM, supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were infected at various multiplicities of infection (MOIs) for up to 72 h. Culture media samples were assayed for mGH using the RIA procedure described below for serum samples. To assess the electrophoretic characteristics of mGH secreted into serum-free culture media, aliquots were precipitated with ice-cold ethanol, subjected to SDS-PAGE (10, 14), transferred to nitrocellulose membranes (Hybond ECL, Amersham, Arlington Heights, IL), and incubated with polyclonal antirat GH serum (lot no. AFP-411S, generously provided by the National Hormone and Pituitary Program, Harbor-UCLA Medical Center).

In vivo gene transfer

All animals were treated according to procedures approved by the National Institute of Dental and Craniofacial Research Animal Care and Use Committee. Anesthesia for all gene transfer procedures was obtained by im injection of ketamine chloride (60 mg/kg) and xylazine (5 mg/kg), as previously described (9, 10). Dwarf mice (6–7 wks old) and their normal litter mates were obtained from Jackson Laboratories (Bar Harbor, ME). Wistar-derived rats (3–4 wks old) were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Animals were allowed to acclimate to our facility for approximately 1 week. Thereafter, each animal received 5×10^9 plaque-forming units (pfu) of either AdCMVmGH or the control virus, Addl312 (a generous gift of Dr. T. Shenk, Princeton University, Princeton, NJ). Virus typically was suspended in 25–50 μl of vehicle [10 mM Tris (pH 7.4), 0.1 mM $MgCl_2$, 10% glycerol] and administered either by im injection (quadriceps), by iv infusion (femoral vein), or by submandibular duct cannulation, as described previously (10). For all animals studied at the 4-day time point, an injection of dexamethasone (4 mg/kg) was administered at the time of gene transfer and the following 3 days, to limit the host immune response (16). At the conclusion of these experiments, animals were euthanized by CO_2 gas, and blood samples were obtained from the tail vein. For all animals followed for 70 days, dexamethasone was administered as above, but blood was obtained from the thoracic cavity after euthanasia.

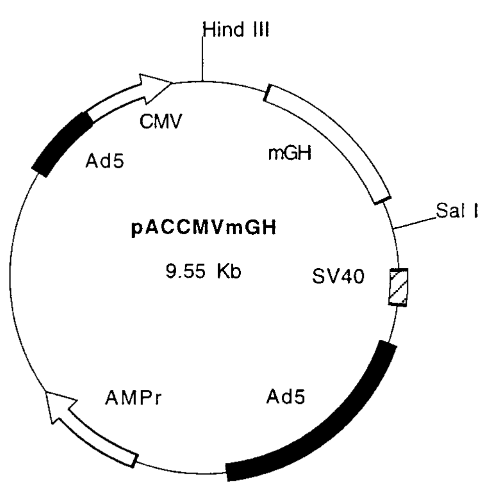

FIG. 1. Shuttle vector constructed with mGH cDNA. As described in *Materials and Methods*, pACCMVmGH was constructed using pACCMV-pLpA and the mGH cDNA. CMV indicates the cytomegalovirus promoter/enhancer; mGH indicates the transgene cDNA; and SV40, the SV40 polyadenylation sequence; AMPr, the ampicillin resistance gene; and Ad5, type 5 adenovirus sequences.

RIA of sera and serum chemistry analyses

Mouse and rat sera were prepared from blood samples obtained as above. For mGH determination with sera and culture media (above), a double-antibody RIA was used, including highly purified mouse pituitary GH (lot no. AFP-10783B) as tracer and reference, and antiserum to rat GH (lot no. AFP-411S) at a dilution of 1:3,000,000. This RIA is specific for mGH and unreactive with other hormones of the mouse pituitary gland. The within-assay coefficient of variation was 4.7%, and the between-assay coefficient of variation was 7.4%. For assay of rat GH, purified rat pituitary GH (lot no. AFP 3190B) was used as tracer and reference, as was the above antiserum to rat GH (lot no. AFP-411S), also at 1:3,000,000. In our assay for mouse GH, both rat and mouse GH are nearly equally reactive. These immunoreagents are distributed to all requestors by the National Hormone and Pituitary Program, Harbor-UCLA Medical Center, Torrance, CA (FAX: 310-222-3432). Insulin-like growth factor 1 (IGF-1) levels in rat sera were determined by RIA (Endocrine Sciences, Inc., Calabasas Hills, CA). Routine rat serum chemistry parameters also were assessed (ANTEC Diagnostics, Farmingdale, NY).

Histological analyzes of growth plates

Bones were obtained from dwarf animals and normal litter mates, fixed for 16–24 h in 4% paraformaldehyde, and then demineralized for 7–10 days in 10% EDTA. After dehydration and embedding in paraffin, the bones were sectioned at 5 μm and stained with hematoxylin/eosin.

Statistical analyses

Results obtained from *in vivo* experiments were tested for statistical significance using a *t* test (for parametric data) or a Mann-Whitney *U* test (for nonparametric data).

Results

Characterization of mGH expression in vitro

As described in *Materials and Methods*, we constructed a replication-deficient, recombinant adenovirus-encoding mGH, AdCMVmGH. To test the ability of this virus to direct the expression of mGH, we infected an epithelial cell line (SMIE), which normally does not make GH. As shown in Fig. 2, we analyzed the culture media using a quantitative RIA procedure. Cells were infected with different MOIs, from 0–300, and the culture media was assayed for mGH at different time points up to 72 h (Fig. 2). For all positive media samples, the slopes of the generated RIA dilution curves were parallel to those determined with authentic mGH standards (data not shown). Production of mGH by SMIE cells was dependent on AdCMVmGH dose and increased with time. Little-to-no mGH was expressed at MOIs less than 10. At higher viral doses, substantial levels of mGH were detected. For example, at 72-h postviral infection, the culture media contained 6.7 ± 0.3, 46.2 ± 1.8, and 183.7 ± 6.5 ng/ml mGH for cells infected at MOIs equal to 10, 100, and 300, respectively. The presence of mGH in the culture media was also demonstrated by Western blot analysis (not shown).

Efficacy of mGH expression in vivo

To test the *in vivo* efficacy (and thus, the potential experimental utility) of AdCMVmGH, we administered the virus either via a single im injection (quadriceps) or into the submandibular duct of Snell dwarf mice. As a control for virus administration, we injected some animals with Addl312, a similar replication-deficient adenovirus but without any transgene. As shown in Fig. 3, which presents serum levels of mGH, the dwarf mice used in these experiments endogenously expressed very low levels of GH (1.08 ± 0.06 ng/ml), whereas their normal litter mates express considerably higher levels (20.35 ± 22.9 ng/ml). Intramuscular administration of AdCMVmGH to eight dwarf mice resulted in an elevation of serum mGH levels, in all animals, to values significantly greater then those receiving the control vector, (range: 3.3–249.2 ng/ml; mean ± SE, 42 ± 29.7; Mann-Whitney test, $P < 0.002$) when measured 4 days post injection. Conversely, GH values were not significantly elevated after im AdCMVmGH administration to normal animals, compared with that observed after Addl312 administration. Submandibular administration of AdCMVmGH was attempted in four dwarf mice. Despite the small size of these animals, submandibular glands were successfully cannulated in three of four mice, resulting in serum mGH levels (64.1, 3.4, 11.0 ng/ml) well above that found in control dwarf mice (see above).

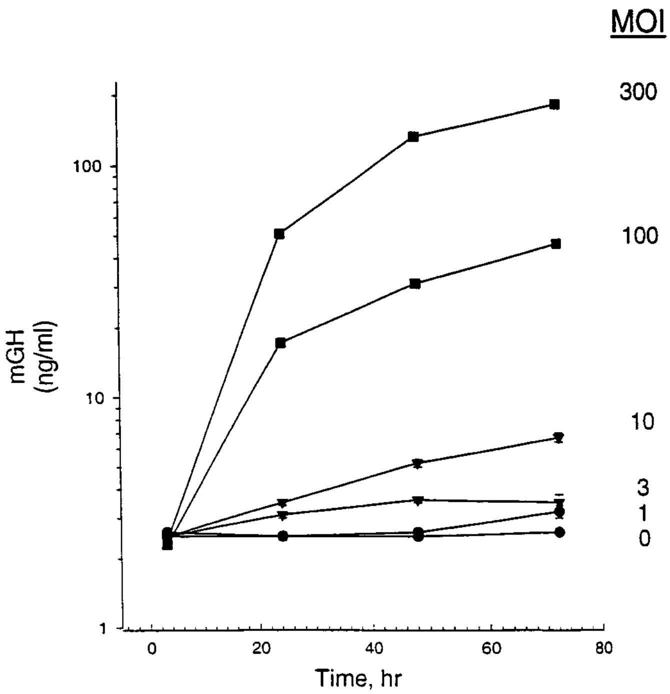

FIG. 2. mGH production in SMIE cells *in vitro*. SMIE cells were infected with different doses of AdCMVmGH (MOI = 0–300) for up to 72 h. Medium was assayed for mGH as described in *Materials and Methods*. Data shown are the mean ± SE for three separate determinations. Where *error bars* are not shown, they are smaller than the symbol.

Biological responses to mGH

As an initial test of the biological activity of the mGH directed by AdCMVmGH, we used a global measurement parameter, animal body weight gain (Fig. 4). Normal mouse litter mates weighed approximately 25–30 g, and im injection of either Addl312 or AdCMVmGH had no effect on their body weight over the 4-day experimental period. Dwarf mice typically weighed about one fourth to one third the size of their normal litter mates (~6–9 g). Injection of AdCMVmGH, however, resulted in a highly significant ($P = 0.01$) increase in the weight of the dwarf mice (range, 2.5–15.1%; mean ± SE, 8.15 ± 1.6%), compared with that seen when the mice were injected with Addl312 (−1.55 ± 3.0). In a second experiment, dwarf mice also received a single im dose of either Addl312 or AdCMVmGH (5×10^9 pfu) but were followed for more than 2 months. As shown in Fig. 5, the animals injected with AdCMVmGH grew in an almost linear fashion for up to 30 days and plateaued thereafter. Conversely, animals injected with Addl312 showed essentially no weight gain

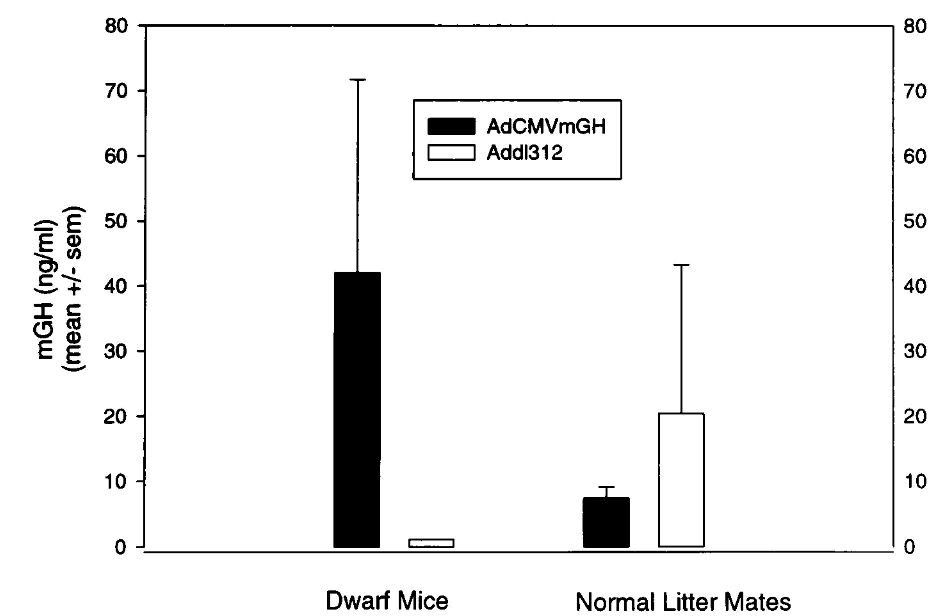

FIG. 3. Effect of im AdCMVmGH administration on mGH levels in the serum of dwarf mice. Dwarf mice or their normal litter mates were injected with either AdCMVmGH or Addl 312 (control virus), at 5×10^9 pfu/animal. After 4 days, sera were obtained and mGH measured, as described in *Materials and Methods*. Data shown are the mean ± SE of results from four to eight animals. Differences seen in dwarf mice are significant, by Mann-Whitney test ($P < 0.002$), whereas differences between normal litter mates are not significant.

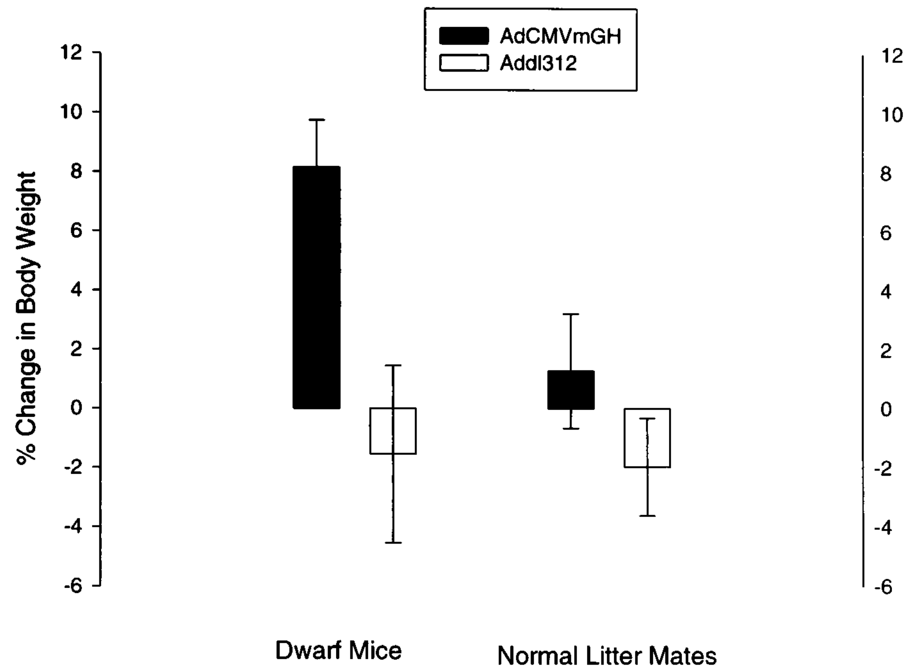

FIG. 4. Effect of im AdCMVmGH administration on body weight in dwarf mice. Animals were infected with either AdCMVmGH or Addl 312, as described in Fig. 3. Animals were weighed at the start of the experiment and after 4 days. Data are the mean ± SE of results from four to eight animals. Differences seen in dwarf mice are significant, by Mann-Whitney test ($P = 0.01$), whereas differences between normal litter mates are not significant.

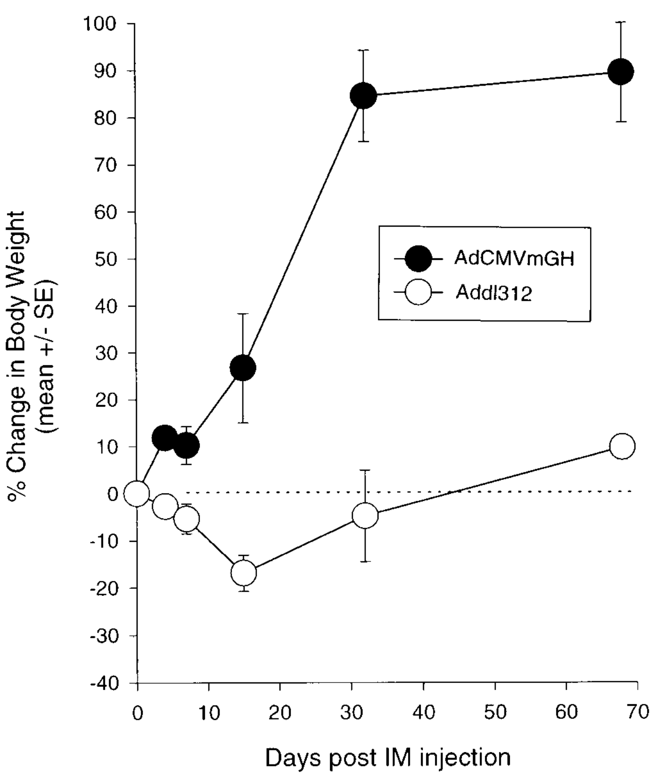

FIG. 5. Effect of im AdCMVmGH administration on the time course of body weight gain in dwarf mice. Animals were injected with either AdCMVmGH or Addl312, as described in Fig. 4, and were weighed periodically for up to 70 days. Animals were killed at day 70, and serum was obtained for mGH determination. Data are the mean ± SE of results from three (AdCMVmGH) or four (Addl312) animals.

over the experimental period. At time of sacrifice, animals injected with AdCMVmGH had 2-fold higher serum GH levels than the control animals (4.2 ± 0.1 *vs.* 2.0 ± 0.4 ng/ml; $t = 4.7$, $P = 0.005$).

Because of blood volume limitations, we were able to obtain only enough serum for measurement of mGH from dwarf mice. To assess several metabolic parameters that may be altered by the biological activity of GH, we administered AdCMVmGH to young rats (3–4 wks old) via an iv route. This route of recombinant adenoviral administration in rats primarily leads to transduction of hepatocytes in the liver (17) and marked transgene expression. Table 1 provides data from these experiments in which sera were obtained 4 days post injection. When compared with animals injected with the control virus Addl312, injection of AdCMVmGH (on average) led to an increase (>10-fold) in serum GH in these animals (mouse and rat GH were indistinguishable with our RIAs). Four of the six animals had dramatic elevations in GH (>375 ng/ml). Further, serum IGF-1 levels were increased approximately 35%; serum cholesterol, approximately 60%; and serum triglycerides, approximately 40%. No significant changes were detected in serum glucose or serum protein levels or in the BUN:creatinine ratio.

The growth plate, a primary target of GH, also was examined in dwarf mice and their normal litter mates. Dwarf mice exhibit an expanded growth plate, marked by an overt delay in maturation with a poorly developed site of secondary ossification of the epiphysis, and reduced bone formation in the metaphysis (18). We observed no consistent changes in the growth plates of dwarf mice 4 days after im administration of AdCMVmGH (not shown).

Discussion

This study demonstrates that a recombinant adenovirus can direct the *in vivo* expression of biologically-active mGH. As judged by electrophoretic and immunochemical characteristics, the transgene product seemed identical to authentic GH. Most importantly, when the virus was administered to dwarf mice deficient in GH, functional mGH production was observed. Serum GH levels exceeded normal levels, and the

TABLE 1. Effect of AdCMVmGH on serum chemistry parameters in 3- to 4-week-old rats

	GH (ng/ml)	IGF-1 (ng/ml)	Glucose (mg/dl)	Protein (g/dl)	BUN/creatinine	Cholesterol (mg/dl)	Triglycerides (mg/dl)
Control	29.4 ± 5.8	489.0 ± 61.1	332.2 ± 39.1	5.4 ± 0.7	31.8 ± 3.7	77.7 ± 11.7	206.5 ± 28.1
AdCMVmGH	350.3 ± 96.3	673.3 ± 50.7	363.7 ± 33.1	6.35 ± 0.3	32.3 ± 1.9	126.0 ± 14.1	296.5 ± 18.7
	[0.002]	[0.043]	[NS]	[NS]	[NS]	[0.025]	[0.024]

Data represent the mean ± SE for results obtained with six animals in each group. Experiments were performed as described in the text. Numbers in *brackets* at *bottom* of table represent probability values; NS, Not significant. GH values were tested by a Mann-Whitney test; all other values, by a *t* test.

dwarf mice gained an average of approximately 8% of their pretreatment body weight in just 4 days, and nearly doubled their body weight in 30 days. We did not, however, see significant changes in the growth plates of dwarf mice at the 4-day time point. Though this may reflect an insufficient time period of elevated GH exposure, it also may reflect the effects of dexamethasone administration on the skeleton. Because adenoviral vectors elicit a potent immune response, the administration of antiinflammatory drugs is required (16).

This study additionally shows that AdCMVmGH can be successfully and efficiently delivered to rodents *in vivo* using three different routes. The primary delivery site for AdCMVmGH in our *in vivo* dwarf mouse experiments was the quadriceps muscle. We also presented one set of experiments conducted in young (3–4 week) rats, in which the virus was administered via the femoral vein, a delivery route leading primarily to transduction of the liver in rats (17). The iv administrations generally led to high serum GH levels in animals and several significant serum chemistry changes consistent with systemic mGH action and anabolic effects. Additionally, we administered AdCMVmGH via retrograde intraductal instillation after successful cannulation of the submandibular glands of dwarf mice. In three of four dwarf mice, submandibular glands were successfully transduced, leading to serum mGH levels comparable with those seen after im injection.

Previously, we have shown that rat salivary epithelial cells readily can be infected *in vivo* by recombinant adenoviruses using an intraductal route of delivery (19). Furthermore, we have shown that salivary glands can secrete adenoviral-encoded transgene products in an endocrine manner (9, 10). Recently, Goldfine and colleagues (20) reached similar conclusions. They have shown that rodent salivary glands can be transduced by direct administration of plasmid DNA, albeit at much lower levels than seen with adenoviruses. In their studies, hGH was secreted from salivary glands into the bloodstream (20).

The initial choice of muscle as a primary target site was, in part, driven by practical issues of size with the dwarf mice. However, skeletal muscle has emerged as an extremely useful target site for the delivery of circulating proteins (21), allowing fairly stable transgene expression after a single injection of a first-generation recombinant adenovirus (months, to >1 yr) in rodents and nonhuman primates (7, 8). Furthermore, in immunocompetent animals, such as those used herein, the host immune response to recombinant adenoviruses administered in muscle seems to be somewhat milder than that observed when the virus infects other sites (4, 5, 7). Nonetheless, there are several examples of the prolonged (months) expression of a circulating transgene-directed protein after the iv administration of a first-generation adenoviral vector in immuocompetent animals (*e.g.* Refs. 22–24). In the present study, after 70 days, mice injected with AdCMVmGH had average GH levels that were approximately 2-fold higher than mice that received Addl312.

In summary, we have constructed an adenoviral vector that directs the functional expression of mGH at different tissue sites *in vivo*. Given the increased clinical interest in GH therapy for adult GH deficiency, age-associated decreases in bone mass, and cardiac performance (25–28), as well as congenital GH deficiency, we anticipate that AdCMVmGH would be a valuable experimental tool for mouse model, preclinical studies.

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